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Phenotypic and Transcriptomic Analyses Demonstrate Interactions between the Transcriptional Regulators CtsR and Sigma B in *Listeria monocytogenes*▽†

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***Listeria monocytogenes* σ^B positively regulates the transcription of class II stress response genes; CtsR negatively regulates class III stress response genes. To identify interactions between these two stress response systems, we constructed *L. monocytogenes* Δ*ctsR* and Δ*ctsR* Δ*sigB* strains, as well as a Δ*ctsR* strain expressing *ctsR* in *trans* under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter. These strains, along with a parent and a Δ*sigB* strain, were assayed for motility, heat resistance, and invasion of human intestinal epithelial cells, as well as by whole-genome transcriptomic and quantitative real-time PCR analyses. Both Δ*ctsR* and Δ*ctsR* Δ*sigB* strains had significantly higher thermotolerances than the parent strain; however, full heat sensitivity was restored to the Δ*ctsR* strain when *ctsR* was expressed in *trans*. Although log-phase Δ*ctsR* was not reduced in its ability to infect human intestinal cells, the Δ*ctsR* Δ*sigB* strain showed significantly lower invasion efficiency than either the parent strain or the Δ*sigB* strain, indicating that interactions between CtsR and σ^B contribute to invasiveness. Statistical analyses also confirmed interactions between the *ctsR* and the *sigB* null mutations in both heat resistance and invasion phenotypes. Microarray transcriptomic analyses and promoter searches identified (i) 42 CtsR-repressed genes, (ii) 22 genes with lower transcript levels in the Δ*ctsR* strain, and (iii) at least 40 genes coregulated by both CtsR and σ^B, including genes encoding proteins with confirmed or plausible roles in virulence and stress response. Our data demonstrate that interactions between CtsR and σ^B play an important role in *L. monocytogenes* stress resistance and virulence.**

Listeria monocytogenes is a gram-positive facultative intracellular pathogen that can cause severe invasive disease in humans, as well as in a number of different animal species. The vast majority of human listeriosis cases are caused by consumption of contaminated food products (44). The capacity of *L. monocytogenes* to survive and multiply under a wide range of environmental-stress conditions appears to be critical for foodborne transmission of the pathogen (18). Accordingly, a number of transcriptional regulators important for stress response and virulence gene expression have been identified in the organism (25, 40, 49). Positive regulatory factor A (PrfA), which activates the transcription of several *L. monocytogenes* virulence genes, was the first transcriptional regulator identified in the bacterium (40). Subsequently, a number of other *L. monocytogenes* transcriptional regulators have been identified, including four alternative sigma factors (σ^C appears to be present only in *L. monocytogenes* strains classified in lineage II, however) (31, 68), two negative regulators (HrcA and CtsR) involved in the regulation of heat shock genes (25, 49), and a number of two-component regulatory systems (64), as well as other regulators (e.g., CodY [2]). While many of these regulators appear to be important predominantly for transcription of stress response genes, null mutations in some stress response regulators have also been shown to result in reduced virulence or virulence-associated characteristics (6), thus providing evidence of mechanistic links between stress response and virulence in *L. monocytogenes* (29).

The stress-responsive alternative sigma factor σ^B appears to have a central role in coordinating *L. monocytogenes* stress response and virulence gene expression (30, 32, 43, 48). σ^B directly regulates the transcription of a large regulon in *L. monocytogenes* (30) by associating with the RNA polymerase core enzyme, thereby allowing the initiation of transcription of genes preceded by σ^B-dependent promoters. *L. monocytogenes* σ^B contributes to survival under a variety of conditions, including acid and oxidative stresses and during carbon starvation (1, 18, 47). In addition to regulating the expression of stress response genes, σ^B also activates the transcription of virulence genes, such as *inlAB* and *bsh* (30). Some virulence genes (e.g., *inlA* and *inlB*) are coregulated by both PrfA and σ^B (30, 32, 43, 46). Further, σ^B directly regulates the transcription of *prfA* (48, 56, 57). As both σ^B and PrfA also autoregulate their own transcription (45, 56), the regulatory network contributing to virulence gene expression involves at least two transcriptional regulators. While initial characterization of this regulatory network, including through array-based characterization of the σ^B and PrfA regulons, has been performed (30, 32, 46), our understanding of the contributions of other regulators to this and other stress response and virulence gene transcription networks is limited. Initial evidence from our group suggested

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TABLE 1. *L. monocytogenes* strains used in this study

Strain	Characteristics	Source or reference
10403S	Parent strain, serotype 1/2a	3
FSL A1-254	Δ*sigB*	63
FSL H6-190	Δ*ctsR*	This study
FSL H6-193	Δ*ctsR* Δ*sigB*	This study
FSL H6-195	Δ*ctsR* tRNA^{Arg}::pLIV2 *ctsR-mcsA*	This study

TABLE 2. PCR primers used in this study

Primer	Sequence (5′→3′)
ctsR SOE A[a,b]	GGGGTACCTAGGTTTGGAGTGGA AGTG
ctsR SOE B[a,c]	GTCATCACACCTTAATCATACATTAT AAGGAATCTCCTTTC
ctsR SOE C[a]	TATGATTAAGGTGTGATGAC
ctsR SOE D[a,d]	GCTCTAGACGCTCGTTCAACTTCTGC
YWH1 ctsR A[e]	ATCTAAGCGTGAAGCCCC
YWH2 ctsR B[e]	ATCACGGACAACAGCAGC
IPTG ctsR primer A[f]	CGGGATCCTTGCAAAAAACAGAAAG GAGATTC
IPTG ctsR primer B[f]	AACTGCAGCTAACCAAGAACTAAGC CGCG
SRM10 pLIV2 R[g]	CTATAATAGAAGGTATGGAGG
SRM11 pLIV2 F[g]	CTTTATCTACAAGGTGTGGC

[a] Primer used to generate the *ctsR* deletion allele using SOE PCR.
[b] The KpnI restriction site incorporated into this primer to facilitate cloning is underlined.
[c] The overhang complementary to primer ctsR SOE C is underlined.
[d] The XbaI restriction site incorporated into this primer to facilitate cloning is underlined.
[e] Primer used to confirm construction of the *ctsR* deletion mutant, including sequencing of *ctsR* in the Δ*ctsR* strain (FSL H6-195).
[f] Primer used to generate a fragment containing the upstream *ctsR* ribosome-binding site, *ctsR*, and *mcsA*; the BamHI and PstI restriction sites incorporated into YWH1 ctsR primer A and YWH2 ctsR primer B, respectively, are underlined.
[g] Primer used to confirm construction of pLIV2 *ctsR-mcsA*.

interactions between the transcriptional regulators CtsR and σ^B in *L. monocytogenes*. Specifically, the CtsR-dependent *clpC* showed σ^B-dependent transcription under energy stress, and a putative σ^B-dependent promoter was identified upstream of the *L. monocytogenes ctsR-mcsA-mcsB-clpC* operon (8). In addition, *clpC* and *clpP* are regulated by both σ^B and CtsR in *Bacillus subtilis* (22, 37), a close relative of *L. monocytogenes*.

While σ^B is recognized as a positive regulator of transcription, CtsR (for *c*lass *t*hree *s*tress gene *r*epressor) is a transcriptional repressor. CtsR is active as a dimer and has three different functional domains, including a helix-turn-helix DNA-binding domain, a dimerization domain, and a putative heat-sensing domain (12). In *L. monocytogenes*, CtsR negatively regulates *clp* genes (*clpP*, *clpE*, *clpB*, and *clpC*) at the transcriptional level by binding directly to a heptanucleotide repeat sequence (A/GGTCAAANANA/GGTCAAA) (5, 49, 51); *clp* genes encode general stress proteins, with some genes possibly contributing to *L. monocytogenes* virulence (50). Characterization of *L. monocytogenes* strains with naturally occurring mutations in *ctsR* showed increased resistance of these strains to heat, H_2O_2, and high pressure, consistent with a negative-regulator role for CtsR (27–29) and suggesting that CtsR regulates the transcription of genes important for stress resistance. A naturally occurring *ctsR* mutant also showed decreased resistance to nisin (27). CtsR-dependent transcription of the heat shock gene *clpB* was reported in a *ctsR* null mutant (5), further confirming the importance of CtsR for heat shock. Characterization of another *ctsR* null mutant also showed reduced growth in tissue culture cells, suggesting CtsR contributions to virulence (6).

To examine the contributions of CtsR to stress response and virulence and to explore interactions between CtsR and σ^B, we created three *L. monocytogenes* strains, including a Δ*ctsR* strain, a Δ*ctsR* Δ*sigB* strain, and a Δ*ctsR* strain with *ctsR* fused to an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter and integrated at a tRNA^{Arg} locus. These strains, in combination with a Δ*sigB* strain (63) and the parent strain, were used for phenotypic and microarray-based analyses.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. monocytogenes* 10403S and isogenic mutants of this parent strain were used in this study (Table 1). Stock cultures were stored at −80°C in brain heart infusion (BHI) (Difco, Sparks, MD) broth supplemented with 15% glycerol and streaked onto BHI agar plates prior to each experiment. For most experiments, overnight bacterial cultures were grown in BHI broth at 37°C with shaking (250 rpm), followed by inoculation into 5 ml of BHI broth (1:100 dilution) and growth at 37°C with shaking to an optical density at 600 nm (OD_{600}) of 0.4, followed by another 1:100 dilution into BHI broth with subsequent growth with shaking at 37°C to an OD_{600} of 0.4 (representing log phase) unless otherwise specified. Multiple serial passages were used to generate cultures comprised of synchronized log-phase bacterial cells.

Construction of Δ*ctsR* null mutants. The nonpolar Δ*ctsR* strain (FSL H6-190) (Table 1) was constructed using allelic-exchange mutagenesis as previously detailed (7). Briefly, splicing by overlap extension (SOE) PCR (Table 2 lists the primers used) was used to construct a Δ*ctsR* allele with an in-frame 447-bp deletion within the *ctsR* open reading frame (ORF), which was cloned into pKSV7, yielding plasmid pUS-1. This plasmid was electroporated into *L. monocytogenes* 10403S, and transformants were serially passaged at 41°C in BHI with chloramphenicol (10 μg/ml) to select for cells in which the plasmid had integrated into the chromosome by homologous recombination. Colonies obtained during subsequent passages at 30°C in BHI without chloramphenicol were screened for chloramphenicol sensitivity (indicating a second homologous-recombination event with loss of pUS-1). Chloramphenicol-sensitive isolates were then screened by PCR to identify isolates with the Δ*ctsR* allele. The pUS-1 plasmid construct was also electroporated into a Δ*sigB* strain (FSL A1-254) to construct a Δ*ctsR* Δ*sigB* mutant (FSL H6-193) (Table 1) by the approach described above. Allelic-exchange mutations for Δ*ctsR* and Δ*ctsR* Δ*sigB* strains were confirmed by PCR amplification and direct sequencing of the PCR product with the external primers YWH1 ctsR A and YWH2 ctsR B (Table 2).

Construction of *L. monocytogenes* expressing *ctsR* from an IPTG-inducible promoter. Plasmid pLIV2 (26), obtained from D. Higgins (Harvard Medical School, Cambridge, MA), was used to construct an *L. monocytogenes* Δ*ctsR* strain expressing *ctsR-mcsA* under the control of the IPTG-inducible P_{spac} promoter present in pLIV2. Plasmid pLIV2 is derived from pLIV1 (10) and the site-specific phage integration vector pPL2 (39); the pPL2-derived PSA bacteriophage integrase gene (PSA int) and attachment site (attPP′) present in pLIV2 allow site-specific integration of this plasmid into the *L. monocytogenes* tRNA^{Arg} gene. We PCR amplified a fragment containing *ctsR* and *mcsA* (Table 2 lists the primers used), including the upstream *ctsR* ribosome-binding site, from *L. monocytogenes* 10403S and cloned the PCR product into pLIV2, generating plasmid pYWH-7. The DNA sequence of the plasmid insert was confirmed by sequencing. Plasmid pYWH-7 was electroporated into the *L. monocytogenes* 10403S Δ*ctsR* strain, followed by selection for plasmid integration on BHI agar plates containing 10 μg/ml chloramphenicol, yielding a Δ*ctsR* tRNA^{Arg}::pLIV2 *ctsR-mcsA* strain (FSL H6-195) (Table 1), which contained a single copy of pLIV2 with *ctsR-mcsA* under the control of an IPTG-inducible P_{spac} promoter. Site-specific chromosomal integration was confirmed by PCR amplification using primers NC16 and PL95 (39). Addition of 0.5 mM IPTG to BHI was used to activate the transcription of *ctsR-mcsA* in strain FSL H6-195.

Swarming behavior. To evaluate swarming behavior, single colonies of the parent or mutant strains were used to stab inoculate tryptic soy broth agar plates containing 0.4% agar, which were then incubated at room temperature for 48 h.

Swarming ability was assessed by measuring the radius of the colony, which was then normalized to the colony radius for the parent strain (*L. monocytogenes* 10403S). Three independent assays were performed for each strain tested. An isogenic *L. monocytogenes* Δ*flaA* mutant (55) was included as a negative control in each assay.

Heat survival experiments. Heat survival experiments were conducted to evaluate the heat resistance of the parent strain, 10403S, as well as Δ*ctsR*, Δ*sigB*, Δ*ctsR* Δ*sigB*, and Δ*ctsR* $tRNA^{Arg}$::pLIV2 *ctsR-mcsA* strains. The parent strain and the Δ*ctsR* $tRNA^{Arg}$::pLIV2 *ctsR-mcsA* strain were also grown with and without the addition of IPTG. The heat survival experiments were performed using a continuous microflow apparatus, as previously described (41), to allow reproducible short-time exposure to heat stress. Briefly, log-phase cells (OD_{600} = 0.4) were pumped from a 50-ml side arm flask through a microcoil that was submerged in an insulated 72°C oil bath (Virtis Research Equipment, Gardiner, NY). The cells were kept at 72°C for either 4 or 8 s, followed by collection of the heat-treated cells using a sterile vial. Bacterial numbers were determined by spread plating the bacteria on BHI agar prior to and after heat treatment. Heat survival was expressed as a log reduction value, which was calculated by subtracting the bacterial numbers (in log CFU/ml) after heat treatment from the bacterial numbers (in log CFU/ml) before heat treatment. Three independent experiments were performed.

Invasion assays. Invasion efficiency in Caco-2 cells was determined for the parent strain, as well as for Δ*ctsR*, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains that were either (i) grown to log phase (OD = 0.4) at 37°C or (ii) grown to log phase, followed by exposure to BHI with 0.3 M NaCl for 10 min at 37°C. Exposure to 0.3 M NaCl was performed to induce σ^B activity (61), thus enabling enhanced detection of CtsR-σ^B interactions. Invasion assays were also performed with *L. monocytogenes* strains grown to early stationary phase (i.e., growth to an OD_{600} of 1, followed by an additional 3 h of incubation) at 30°C, as *L. monocytogenes* grown at that temperature shows increased motility compared to bacteria grown at 37°C. Although many *L. monocytogenes* isolates are nonmotile at 37°C and motile at 30°C, strain 10403S is motile at 37°C, although at a reduced level compared to cells grown at 30°C (24, 62).

Invasion assays in Caco-2 cells (ATCC HTB-37) were performed as previously described (53). For all invasion assays, confluent Caco-2 monolayers grown in 24-well plates were inoculated with 10 μl *L. monocytogenes* cells (three wells/strain). After incubation for 30 min, the Caco-2 cells were washed three times with phosphate-buffered saline, and at 45 min postinoculation, fresh medium containing gentamicin (150 μg/ml) was added to kill extracellular bacteria. At 90 min postinoculation, the Caco-2 cells were washed three times with phosphate-buffered saline and then lysed by the addition of ice-cold sterile distilled water, followed by vigorous pipetting. Intracellular *L. monocytogenes* numbers were determined by spiral plating lysed Caco-2 cell suspensions on BHI agar using an Autoplate 4000 spiral plater (Spiral Biotech Inc., Norwood, MA). The invasion efficiency was calculated as the number of intracellular bacteria recovered (in CFU) relative to the bacterial numbers (in CFU) used for the inoculation; invasion efficiencies for mutant strains were then normalized to the 10403S parent strain invasion efficiency, which was set as 100%. Three independent invasion assays were performed for each *L. monocytogenes* strain tested.

TaqMan qRT-PCR. RNA isolation for quantitative real-time (qRT) PCR was performed as previously described (42). qRT-PCR with previously described primers and probes (32, 34, 57, 61) was used to measure transcript levels for *inlA*, *clpC*, *gadA*, *prfA*, and *plcA*, as well as for two housekeeping genes (*rpoB* and *gap*). RNA was isolated from the parent strain, and selected mutants (Δ*ctsR*, Δ*ctsR* Δ*sigB*, and Δ*sigB*) that were either (i) grown to log phase (OD_{600} = 0.4) at 37°C or (ii) grown to log phase, followed by exposure to BHI with 0.3 M NaCl for 10 min at 37°C. qRT-PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), as detailed previously (42, 61). Reverse transcriptase negative control reactions, DNA standard curves, and analysis of qRT-PCR were performed essentially as described previously (42). Absolute cDNA copy numbers, calculated based on DNA standard curves, reflect mRNA levels for each target gene present in each RNA sample. Relative cDNA copy numbers were calculated as log cDNA copy numbers normalized to the geometric mean of cDNA copy numbers for the housekeeping genes *rpoB* and *gap* {i.e., $\log_{10}$ target gene − [($\log_{10}$ *rpoB* + $\log_{10}$ *gap*)/2]}). qRT-PCR was repeated three times using three independent RNA isolations from cells grown on three different days (42).

In addition, qRT-PCR primers and probes were designed for *clpB* and lmo1138 (see Table S1 in the supplemental material). qRT-PCR with these two primer/probe sets was performed using RNA isolated from the *L. monocytogenes* parent and Δ*ctsR* strains (grown to log phase) to confirm CtsR-dependent repression of the two genes.

Microarray-based transcriptomic analyses. To identify CtsR-dependent genes, two separate sets of microarray experiments were performed, including (i) one set of experiments comparing transcript levels between the parent and the Δ*ctsR* strains, both grown to log phase, and (ii) one set of experiments comparing transcript levels between the Δ*ctsR* $tRNA^{Arg}$::pLIV2 *ctsR-mcsA* strain (grown with 0.5 mM IPTG) and the Δ*ctsR* strain, both grown to log phase. Microarray construction, RNA isolation and purification, cDNA labeling, and hybridization were performed as previously detailed (4). Briefly, RNA for microarrays was isolated using the RNAprotect bacterial reagent and the RNeasy Midi kit (Qiagen, Valencia, CA). DNase treatment of RNA was performed essentially as described previously (30), except that 40 U of DNase was used. The isolated RNA (in RNase-free water) was quantified and checked for purity using OD_{260} and OD_{280} measurements performed on a Nanodrop spectrophotometer (Nanodrop Technology Inc., Wilmington, DE). Agarose gel electrophoresis was used to verify RNA integrity.

cDNA was synthesized and differentially labeled using the SuperScript Plus Indirect cDNA Labeling System (Invitrogen, Carlsbad, CA). cDNA was generated from 10 μg of RNA using random primers in an overnight reverse transcription reaction at 42°C. The cDNA was purified using a Qiagen PCR purification kit prior to indirect labeling with Alexa Fluor 555 or Alexa Fluor 647 (performed overnight at room temperature). Labeled cDNA was purified using a Qiagen PCR purification kit to remove any unincorporated dye, and the labeled cDNA was quantified and checked for purity using OD_{260} and OD_{280} measurements.

Microarrays were constructed using 70-mer oligonucleotides targeting 2,857 *L. monocytogenes* ORFs (Qiagen Operon Array-Ready Oligo Sets) identified in the annotated genome sequence for *L. monocytogenes* EGD-e (23). Oligonucleotides (70-mer) targeting five *Saccharomyces cerevisiae* ORFS (*act1*, *mfa1*, *mfa2*, *ras1*, and *ste3*) were included on the microarray to serve as nonhybridizing controls, as described previously (67). Prior to hybridization, the slides were blocked and washed essentially as described by Chan et al. (4).

For each microarray, two target cDNAs (e.g., cDNAs from the parent and the Δ*ctsR* strains) were combined in one tube, dried, and then resuspended in hybridization buffer containing sodium dodecyl sulfide (SDS), SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), salmon sperm DNA, dithiothreitol, and formamide. The combined cDNA target (in a 50-μl volume) was overlaid onto the microarray slides using mSeries LifterSlips (Erie Scientific, Portsmouth, NH). Following overnight hybridization at 42°C, the slides were washed in 2× SSC plus 0.1% SDS for 5 min at 42°C, followed by room temperature washes in 2× SSC plus 0.1% SDS, 2× SSC, and 0.2× SSC for 5 min each. The slides were centrifuged to dry and scanned with a GenePix 4000B Scanner (Molecular Devices, Sunnyvale, CA) at the Microarray Core Facility (Ithaca, NY).

Scanned microarray images were gridded using GenePix Pro 6.0 software. Raw image analysis data were preprocessed, and significant differences in gene expression patterns between strains were determined using LIMMA software (58) from R/BioConductor (21). Following background correction by the normexp method, within-array normalization (print-tip loess, a locally weighted linear regression model) and between-array normalization (scale) were used to correct for spatial and intensity biases and to make the results comparable across arrays. The LIMMA package was also used for differential expression analysis (59) to calculate moderated *t* and *B* statistics and *P* values (adjusted for multiple comparisons by controlling for the false-discovery rate). Genes with an adjusted *P* value of <0.05 were considered statistically significant, and a change of $\geq$1.5-fold was used as a cutoff for identification of differentially expressed genes. Genes that showed significantly different transcript levels either (i) between the parent and the Δ*ctsR* strains or (ii) between the Δ*ctsR* $tRNA^{Arg}$::pLIV2 *ctsR-mcsA* and the Δ*ctsR* strains were considered putative CtsR-dependent genes.

HMM searches. Potential CtsR-binding sites were determined using hidden Markov model (HMM) searches as previously described (30). The HMM training alignments included 33 CtsR-binding operators previously identified in gram-positive bacteria (11). The HMM model was searched against the template and nontemplate sequences for the *L. monocytogenes* EGD-e genome. Outputs were filtered, and only hits within 300 bp upstream of a start codon for an ORF, as annotated by ListiList (http://genolist.pasteur.fr/ListiList), and with an E value of $\leq$0.01 were considered meaningful.

Statistical analysis. All statistical analyses were performed using SAS (SAS online Doc8, version 8; SAS, Inc., Cary, NC). qRT-PCR and heat survival were analyzed using one-way or two-way analysis of variance. For both swarming assays and invasion assays, the phenotypic results for the mutant strains (i.e., swarming ability and invasion efficiency) were normalized to the parent strain's swarming ability or invasion efficiency (which were set at 100%); comparisons between mutant and parent invasion efficiencies were performed using one-sample *t* tests; Bonferroni corrections to the *P* values were used to adjust for

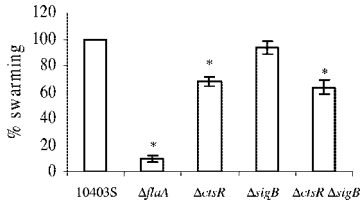

FIG. 1. Swarming abilities of the *L. monocytogenes* 10403S parent strain and Δ*flaA*, Δ*ctsR*, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains. Δ*flaA* was included as a negative control strain. Swarming ability was assessed by stabbing inocula into semisolid agar and then measuring colony growth radii after incubation at room temperature for 48 h; the growth radius for each mutant strain was normalized to the growth radius of the parent strain (10403S), which was set at 100%. An asterisk indicates that the growth radius of that mutant was significantly smaller than the growth radius for the parent strain (as determined by a one-sample *t* test; *P* values were adjusted for multiple testing using a Bonferroni correction). The Δ*ctsR* and the Δ*ctsR* Δ*sigB* strains showed significantly smaller radii than the Δ*sigB* strain and significantly larger radii than the Δ*flaA* strain. The data shown represent the averages of three independent experiments; the error bars indicate standard deviations; no error bar is shown for the parent strain, since the parent strain radius was set at 100% for each experiment.

multiple comparisons. To test whether interactions caused by the deletion of two genes (i.e., *sigB* and *ctsR*) had a significant effect on a given phenotype (e.g., invasion efficiency), a linear model with two factors (gene 1 [*ctsR*] presence/absence and gene 2 [*sigB*] presence/absence) was used. For all tests, statistical significance was established at a *P* value of <0.05; significant *P* values are reported as the actual value, unless *P* was <0.001.

Microarray data accession number. Raw and normalized microarray data in MIAME format are available at the NCBI Gene Expression Omnibus (GEO) data repository (15) under accession number GSE7514.

RESULTS

Loss of CtsR results in reduced swarming ability. Since an *L. monocytogenes* strain with a naturally occurring 3-bp deletion in *ctsR* was reported as having reduced *flaA* transcription and protein expression (29), we examined the swarming ability of our Δ*ctsR* mutant strain relative to that of the Δ*sigB* and Δ*ctsR* Δ*sigB* strains at room temperature (Fig. 1). Both the Δ*ctsR* and the Δ*ctsR* Δ*sigB* strains showed similar swarming, which was significantly lower than the swarming ability observed for the parent strain but also significantly higher than the swarming ability of the Δ*flaA* strain (Fig. 1). The Δ*sigB* strain showed no evidence of reduced swarming ability (Fig. 1).

Loss of CtsR increases *L. monocytogenes* heat resistance. To evaluate the roles of CtsR and σ^B in *L. monocytogenes* heat resistance, the ability of log-phase cells to survive exposure to 72°C for 4 or 8 s was tested for the parent strain and the Δ*ctsR*, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains (Fig. 2). The Δ*ctsR* and the Δ*ctsR* Δ*sigB* strains both showed significantly lower log reductions (i.e., higher heat resistance) than the parent strain after either 4 or 8 s of exposure to 72°C, consistent with the role of CtsR as a negative regulator of stress gene transcription. While the Δ*sigB* strain showed numerically higher log reduction after heat treatment for 4 s (7.66 log units) than the parent strain (6.56 log units; 10403S without IPTG addition), the difference in log reduction was not significant. Interestingly, after exposure to 72°C for 8 s, the Δ*ctsR* Δ*sigB* strain showed higher log reduction values than the Δ*ctsR* strain, further suggesting reduced heat resistance associated with a *sigB* deletion. Formal

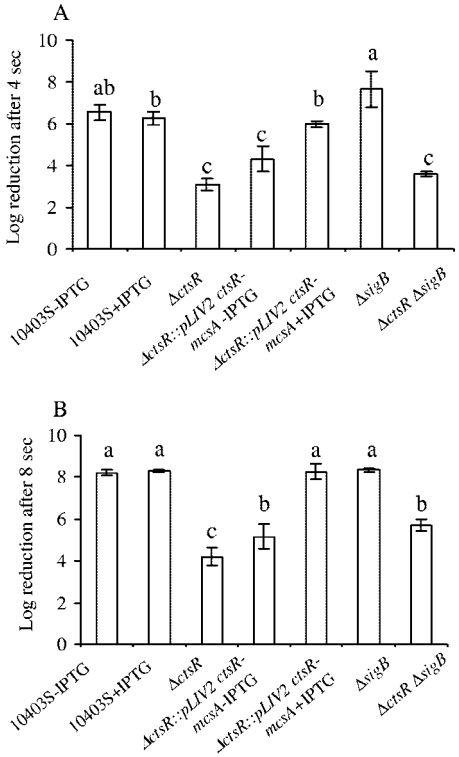

FIG. 2. Log reduction after exposure of the *L. monocytogenes* parent strain (10403S) and Δ*ctsR*, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains to 72°C for 4 s (A) or 8 s (B) and Δ*ctsR* $tRNA^{Arg}$::pLIV2 *ctsR-mcsA* strain grown with (+IPTG) and without (−IPTG) IPTG. The strains were grown to log phase before the heat survival experiments; survival experiments for the parent strain were performed with bacteria grown with (+IPTG) and without (−IPTG) IPTG to ensure that the addition of IPTG to the growth media did not affect heat survival. Strains with higher log reductions showed greater sensitivity to heat (e.g., *L. monocytogenes* 10403S showed an 8.2-log-unit reduction after 8 s of exposure to 72°C, while *L. monocytogenes* Δ*ctsR* showed only a 4.2-log-unit reduction; the Δ*ctsR* strain is thus less heat sensitive than the parent strain, 10403S). Log reduction was calculated by subtracting the bacterial numbers (in log CFU/ml) after heat treatment from the bacterial numbers (in log CFU/ml) before heat treatment. The data shown represent the averages of three independent experiments; the error bars indicate standard deviations. Tukey's multiple-comparison procedure was used to determine whether heat survival differed between specific strains; the bars labeled with different letters indicate log reduction values that differed significantly ($P < 0.05$), while the bars labeled with identical letters indicate log reduction values that did not differ significantly. Statistical analyses of log reduction values for the parent strain (10403S−IPTG) and the Δ*ctsR*, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains using a linear model indicated a significant effect on heat survival of the *ctsR* deletion ($P < 0.001$ for survival after both 4 and 8 s), the *sigB* deletion ($P = 0.0206$ and $P < 0.001$ for survival after 4 and 8 s, respectively), and the interaction between the *ctsR* and *sigB* deletions ($P = 0.0023$ for survival after 8 s).

statistical analyses (using a linear model, as detailed in Materials and Methods) to determine whether the interaction between the *ctsR* and *sigB* null mutations affected heat resistance showed a significant effect of the factor "interaction" on log reduction after 8 s ($P = 0.0023$) but no significant effect of this factor on heat survival after 4 s at 72°C.

To confirm the role of CtsR in *L. monocytogenes* heat shock resistance, we also tested the heat resistance of the Δ*ctsR*

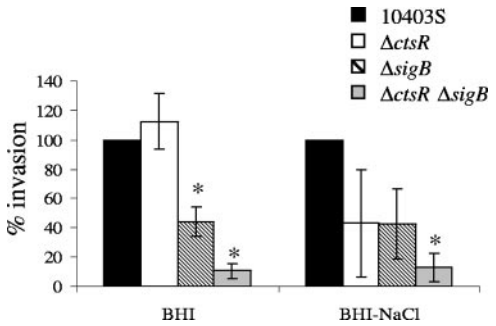

FIG. 3. Invasion efficiency in the human intestinal epithelial cell line Caco-2 of the *L. monocytogenes* parent strain (10403S) and the Δ*ctsR*, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains that were either (i) grown to log phase in BHI at 37°C (BHI) or (ii) grown to log phase, followed by exposure to 0.3 M NaCl for 10 min at 37°C (BHI-NaCl). Invasion efficiency was calculated as the number of intracellular bacteria recovered (in CFU) relative to the bacterial numbers (in CFU) used for inoculation; invasion efficiencies for mutant strains were then normalized to the parent strain invasion efficiency, which was set as 100%. The data shown represent the averages of three independent experiments; the error bars indicate standard deviations; no error bar is shown for the parent strain, since its invasion efficiency was set at 100% for each experiment. An asterisk indicates that the invasion efficiency of that mutant was significantly lower than the invasion efficiency for the parent strain (as determined by a one-sample *t* test; *P* values were adjusted for multiple testing using a Bonferroni correction). For bacteria grown in BHI, invasion efficiencies were significantly lower for (i) the Δ*sigB* strain compared to the Δ*ctsR* strain and (ii) the Δ*ctsR* Δ*sigB* strain compared to the Δ*ctsR* or the Δ*sigB* strain. Analyses of log-transformed nonnormalized invasion efficiency data for the parent strain (10403S) and the Δ*ctsR*, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains using a linear model indicated a significant effect on invasion efficiency of the *ctsR* deletion ($P < 0.001$ and $P = 0.0124$ for BHI and BHI-NaCl, respectively), the *sigB* deletion ($P = 0.0044$ and $P = 0.0172$ for BHI and BHI-NaCl, respectively), and the interaction between the *ctsR* and *sigB* deletions ($P = 0.0019$ for BHI).

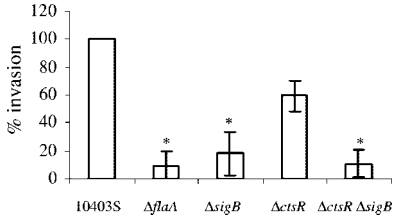

FIG. 4. Invasion efficiencies in Caco-2 cells of the *L. monocytogenes* parent strain (10403S) and Δ*flaA*, Δ*ctsR*, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains that were grown to early stationary phase in BHI at 30°C. Invasion efficiency was calculated as the number of intracellular bacteria recovered (in CFU) relative to the bacterial numbers (in CFU) used for the inoculation; invasion efficiencies for mutant strains were then normalized to the parent strain invasion efficiency (which was set as 100%). The data shown represent the averages of three independent experiments; the error bars indicate standard deviations; no error bar is shown for the parent strain, since its invasion efficiency was set at 100% for each experiment. An asterisk indicates that the invasion efficiency of that mutant was significantly lower than the invasion efficiency for the parent strain (as determined by a one-sample *t* test; *P* values were adjusted for multiple testing using a Bonferroni correction). Statistical analyses of log-transformed nonnormalized invasion efficiency data for the parent strain (10403S) and the Δ*cts*R, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains using a linear model indicated significant effects on invasion efficiency of the *ctsR* deletion ($P = 0.0306$) and of the *sigB* deletion ($P < 0.001$).

$tRNA^{Arg}$::pLIV2 *ctsR-mcsA* strain (grown in the presence or absence of 0.5 mM IPTG), which expresses *ctsR* in *trans* under the control of an IPTG-inducible promoter. The heat survival rates of the 10403S parent strain grown in the absence or presence of IPTG did not differ (Fig. 2), indicating that the mere presence of IPTG in growth media does not affect heat resistance. The log reduction after heat treatment of the Δ*ctsR* $tRNA^{Arg}$::pLIV2 *ctsR-mcsA* strain grown in the absence of IPTG was numerically higher than the log reduction of the Δ*ctsR* strain (Fig. 2), suggesting limited *ctsR* expression in the absence of IPTG, which leads to increased heat sensitivity. The log reduction of the Δ*ctsR* $tRNA^{Arg}$::pLIV2 *ctsR-mcsA* strain grown in the presence of IPTG was similar to the log reduction of the parent strain and significantly higher than the log reduction of the same strain grown in the absence of IPTG. These data indicate that in *trans* expression of *ctsR* fully restores the wild-type phenotype, supporting the idea that the increased heat resistance (i.e., reduced log reduction) of the Δ*ctsR* strain is specifically caused by the *ctsR* deletion.

CtsR and σ^B contribute to invasion efficiency of *L. monocytogenes* grown at 37°C. Invasion efficiencies for Caco-2 cells, a human intestinal epithelial cell line, were initially determined for the *L. monocytogenes* parent strain, as well as for Δ*ctsR*, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains (Fig. 3). For log-phase cells not exposed to salt stress, the parent strain and the Δ*ctsR* strain had similar invasion efficiencies (Fig. 3), while the Δ*sigB* strain showed significantly lower invasion efficiency than both the parent strain and the Δ*ctsR* strain (Fig. 3). Interestingly, the Δ*ctsR* Δ*sigB* strain showed the lowest overall invasion efficiency, which was significantly lower than the invasion efficiency for the Δ*sigB* strain (Fig. 3). The observation that the Δ*ctsR* Δ*sigB* strain had lower invasion efficiency than the Δ*sigB* strain, despite the fact that the Δ*ctsR* strain showed no reduced invasion efficiency (compared to the parent strain), indicated that interactions between the *ctsR* and *sigB* deletions may affect the invasion phenotype. Statistical analysis (using a linear model, as detailed in Materials and Methods) of the log-transformed invasion efficiencies confirmed this and showed a highly significant effect of the interactions between the *ctsR* and *sigB* deletions on the invasion efficiencies of log-phase cells ($P = 0.0019$).

For log-phase cells exposed to 0.3 M NaCl for 10 min, the invasion efficiencies for both the Δ*ctsR* and the Δ*sigB* strains were numerically (but not statistically significantly) lower than for the parent strain (Fig. 3). The Δ*ctsR* Δ*sigB* strain showed the lowest invasion efficiency (significantly lower than the invasion efficiency for the parent strain) (Fig. 3); statistical analyses showed no evidence for significant interactions between the *ctsR* and *sigB* deletions on the invasion efficiencies of log-phase cells exposed to 0.3 M NaCl.

σ^B, and possibly CtsR, contributes to invasion efficiency of *L. monocytogenes* grown at 30°C. Additional invasion experiments in Caco-2 cells were performed using the *L. monocytogenes* parent strain, as well as Δ*flaA*, Δ*sigB*, Δ*ctsR*, and Δ*ctsR* Δ*sigB* strains, grown to stationary phase at 30°C (Fig. 4). Invasion assays with bacteria grown at 30°C were performed, in addition to the invasion assays with bacteria grown at 37°C, as *L. monocytogenes* strain 10403S shows increased motility when grown at 30°C and as our initial experiments (see above) showed that

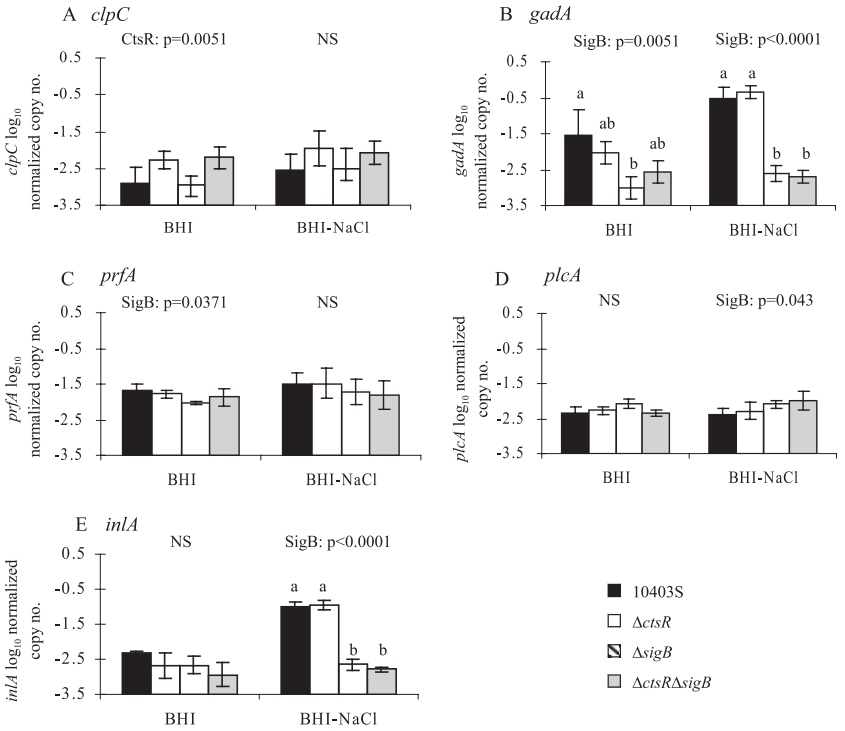

FIG. 5. Transcript levels for *clpC* (A), *gadA* (B), *prfA* (C), *plcA* (D), and *inlA* (E) in the parent strain (10403S) and the Δ*ctsR*, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains. Bacteria were either (i) grown to log phase in BHI at 37°C (BHI) or (ii) grown to log phase, followed by exposure to BHI with 0.3 M NaCl for 10 min at 37°C (BHI-NaCl). Transcript levels are expressed as log cDNA copy numbers normalized to the geometric mean of cDNA copy numbers for the housekeeping genes *rpoB* and *gap* {i.e., $\log_{10}$ target gene − [($\log_{10}$ *rpoB* + $\log_{10}$ *gap*)/2]}; indicated as "$\log_{10}$ normalized copy no." on the *y* axes). The values shown represent the averages of qRT-PCR assays performed on three independent RNA collections; the error bars show standard deviations. NS indicates that neither the *ctsR* deletion, the *sigB* deletion, nor the interaction between the *ctsR* and *sigB* deletions showed a significant effect using a linear model for statistical analyses. If the linear model showed a significant effect of either the *ctsR* deletion or the *sigB* deletion on transcript levels for a given gene for cells grown under a given condition (BHI or BHI-NaCl), the respective *P* value is given on the graph (see Table S2 in the supplemental material for all results of these statistical analyses). Tukey's multiple-comparison procedure was used to determine whether transcript levels for a given gene differed between specific strains; the bars labeled with different letters (a and b) indicate transcript levels that differed significantly ($P < 0.05$), while the bars labeled with identical letters indicate transcript levels that did not differ significantly; if no letters are indicated, no significant differences in the individual comparisons were observed between strains. The linear model may reveal significant effects of a gene deletion, even if transcript levels for the four strains do not differ significantly in the individual comparisons, as the linear model accounts for the effects in the Δ*ctsR* and Δ*sigB* single mutants, as well as in the Δ*ctsR* Δ*sigB* double mutant.

deletion of *ctsR* leads to reduced motility of *L. monocytogenes* 10403S. In these invasion assays with *L. monocytogenes* grown at 30°C, the Δ*flaA* strain (included as a control) showed significantly lower invasion efficiency than the parent strain (Fig. 4), consistent with previous observations of the contributions of flagella to invasion (14, 55). While the Δ*ctsR* strain showed numerically lower invasion efficiency (59.6%) than the parent strain (100%), these differences were only borderline significant ($P = 0.0253$ without a Bonferroni correction; $P = 0.1$ with a Bonferroni correction) (Fig. 4). The Δ*sigB* strain showed reduced invasion efficiency compared to the parent strain, consistent with previous reports (20, 33), and the Δ*ctsR* Δ*sigB* mutant showed significantly lower invasion efficiency than the Δ*ctsR* strain ($P = 0.0005$; *t* test) and numerically, but not significantly, lower invasion efficiency than the Δ*sigB* strain (Fig. 4). Analysis of the log-transformed invasion efficiencies (using a linear model, as described in Materials and Methods) showed significant effects of both the *ctsR* and *sigB* deletions on the invasion efficiencies, further confirming the contributions of both CtsR and σ^B to *L. monocytogenes* invasion efficiency.

qRT-PCR indicates CtsR-dependent *clpC* and σ^B-dependent *prfA*, *plcA*, *inlA*, and *gadA* transcription but no evidence for CtsR and σ^B interactions contributing to transcription of these genes. To evaluate the effects of the *ctsR* deletion, as well as of CtsR-σ^B interactions, on the transcription of selected virulence and stress response genes, qRT-PCR was performed to quantify the transcript levels of *prfA*, *plcA*, *inlA*, *gadA*, and *clpC* in the parent strain, as well as in Δ*ctsR*, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains, either (i) grown to log phase ($OD_{600} = 0.4$) at 37°C or (ii) grown to log phase, followed by exposure to 0.3 M NaCl for 10 min at 37°C (Fig. 5); the same conditions were used to grow cells for invasion assays (see above). These experiments were performed to determine whether interactions between CtsR and σ^B affect the invasion phenotype through contributions to transcriptional regulation of *inlA*, either directly or indirectly (e.g., by affecting the transcription of *prfA*). In addition to *inlA* and *prfA* transcript levels, transcript levels were also determined for *clpC*, *gadA*, and *plcA*, as the transcript levels for these genes serve as indicators for the activities of CtsR (which negatively regulates *clpC*), σ^B (which positively

regulates *gadA*), and PrfA (which positively regulates *plcA*), respectively.

clpC transcript levels were affected by the *ctsR* deletion, with higher *clpC* transcript levels in both the Δ*ctsR* and the Δ*ctsR* Δ*sigB* strains (compared to the parent and Δ*sigB* strains), although the effect of the *ctsR* deletion was only significant for log-phase cells without NaCl exposure (Fig. 5A). These findings are consistent with the previously reported role of CtsR as a negative regulator of the *ctsR-mcsA-mcsB-clpC* operon (49). While previous microarray experiments (55a) (microarray data are available under GEO database accession number GSE7492) showed σ^B-dependent transcription of *mcsA*, *mcsB*, and *clpC* in *L. monocytogenes* log-phase cells exposed to 0.3 M NaCl (see Fig. 7), *clpC* transcript levels were not found to be σ^B dependent here, suggesting that minor differences in environmental-stress exposure protocols may affect σ^B-dependent *clpC* transcription. The notion that σ^B-dependent transcription of *clpC* occurs under specific environmental conditions is also supported by the presence of a σ^B-dependent consensus promoter upstream of *mcsA* and by a previous study that found σ^B-dependent transcription of *clpC* in *L. monocytogenes* exposed to carbonyl cyanide *m*-chlorophenylhydrazone (8).

gadA transcript levels were significantly affected by the *sigB* deletion, regardless of growth conditions (Fig. 5B), and were lowest in the Δ*sigB* and Δ*ctsR* Δ*sigB* strains; effects of the *sigB* deletion were particularly apparent in log-phase cells exposed to NaCl. These findings are consistent with a previous observation that σ^B activity is induced by salt stress and that *gadA* transcription is activated by σ^B (32, 60). *prfA* and *plcA* transcript levels were similar for the parent and the Δ*ctsR*, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains (Fig. 5C and D). Overall statistical analyses indicated significant effects of the *sigB* deletion on *prfA* transcript levels (in log-phase cells), with lower *prfA* transcript levels in the Δ*sigB* strain, consistent with the observation that one of the multiple *prfA* promoters is σ^B dependent (48). Statistical analyses also indicated a significant effect of the *sigB* deletion on *plcA* transcript levels (in log-phase cells exposed to NaCl), with slightly higher transcript levels in the Δ*sigB* and Δ*ctsR* Δ*sigB* strains, although the *P* value was borderline significant ($P = 0.043$). *inlA* transcript levels in log-phase cells were not significantly affected by either the *ctsR* or the *sigB* deletion. *inlA* transcript levels for log-phase cells exposed to 0.3 M NaCl (Fig. 5E) were significantly affected by the *sigB* deletion, with *inlA* transcript levels significantly lower in the Δ*sigB* and Δ*ctsR* Δ*sigB* strain than in the parent and Δ*ctsR* strains. qRT-PCR results showed no evidence that interactions between σ^B and CtsR affect *inlA* transcription under the growth conditions tested here.

Whole-genome microarray analysis identified 64 CtsR-dependent genes. As phenotypic experiments provided evidence that interactions between CtsR and σ^B contribute to both heat resistance and invasion efficiency, we used microarray experiments to characterize the *L. monocytogenes* CtsR regulon. In conjunction with recently completed characterization of the σ^B regulon (55a) (microarray data are available under GEO database accession number GSE7492) with the same Δ*sigB* strain used in all the experiments reported here, an additional goal was to identify genes coregulated by CtsR and σ^B. Microarray experiments to identify the CtsR regulon used comparisons of transcript levels between (i) the parent strain and the Δ*ctsR* strain and (ii) the Δ*ctsR* $tRNA^{Arg}$::pLIV2 *ctsR-mcsA* strain expressing the *ctsR-mcsA* operon in *trans* (grown in the presence of IPTG) and the Δ*ctsR* strain. Genes were classified as CtsR dependent if they showed ≥1.5-fold differences in transcript levels with an adjusted *P* value of <0.05 in either of the two comparisons. Using these criteria, we identified a total of 64 CtsR-dependent genes, including 42 genes negatively regulated by CtsR (i.e., genes that showed higher transcript levels in the Δ*ctsR* strain) (Table 3) and 22 genes that showed lower transcript levels in the Δ*ctsR* strain (Table 4). While all genes that showed lower transcript levels in the Δ*ctsR* strain (indicating positive regulation by CtsR) are likely indirectly regulated by CtsR (as CtsR is a negative regulator), genes that showed higher transcript levels in the Δ*ctsR* strain could be directly or indirectly regulated by CtsR. A total of at least 10 genes appear to be directly regulated by CtsR, as they show higher transcript levels in the CtsR strain, as well as putative or confirmed CtsR-binding sites (Table 5) upstream of a gene or operon. Consistent with a previous report (49), the *ctsR-mscA-mscB-clpC* operon (lmo0229 to lmo0232) was confirmed as directly CtsR repressed. In addition to this operon, four confirmed or putative heat shock genes were also directly repressed by CtsR, including the *clpB*-lmo2205 operon and *clpP*, which all encode traditional class III heat shock proteins, as well as lmo1138, which encodes a protein similar to ClpP (65.4% amino acid similarity to *L. monocytogenes* EGD-e *clpP*). In addition, the *tatAC* operon, which encodes a putative twin argenine translocase secretion system, was found to be directly repressed by CtsR; interestingly, *tatAC* appears to be absent in *L. monocytogenes* serotype 4b strains (13). Surprisingly, *clpE*, which previously has been reported to be CtsR repressed (49), was not found to be CtsR dependent in our microarray studies (*clpE* showed transcript level ratios of −1.34 and −1.24 with an adjusted *P* value of >0.05). CtsR-dependent repression of *clpB* and lmo1138 was also confirmed by qRT-PCR (Fig. 6).

A total of 32 genes appear to be indirectly repressed by CtsR, including *qoxB* and *tpx*, which both encode oxidases. Additional genes indirectly repressed by CtsR include genes encoding proteins that contribute to metabolism (e.g., lmo0811 and lmo1096 [*guaA*]) and putative ABC transporters (e.g., lmo1960 [*fhuC*]), as well as a number of other genes (see Table 3 for a complete list). The 22 genes that appeared to be indirectly upregulated by CtsR (as supported by lower transcript levels in the Δ*ctsR* strain) include a number of genes (e.g., *cheR*, lmo0684, *motA*, lmo0688, *fliM*, and *fliI*) (Fig. 7) located in two large operons (lmo0675 to lmo0689 and lmo0691 to lmo0718) that encode flagellar proteins contributing to motility. Additional genes indirectly upregulated by CtsR encode proteins contributing to acid stress resistance (*gadC* and *gadB*), as well as proteins with roles in metabolism and proteins with unknown functions (Table 4).

The transcription of 40 genes is affected by both CtsR and σ^B, including a number of heat shock genes that are directly regulated by both CtsR and σ^B. Comparison of the CtsR regulon with the *L. monocytogenes* σ^B regulon (55a) (microarray data are available under GEO database accession number GSE7492) identified 40 of the CtsR-dependent genes reported here as also differentially regulated by σ^B (Fig. 7). Nine coregulated genes were positively regulated by σ^B and negatively

TABLE 3. Genes identified by microarray analysis as down-regulated by CtsR[a]

Gene[b]	Differential expression in: *L. monocytogenes* parent strain vs Δ*ctsR*		Δ*ctsR* tRNA^{Arg}::pLIV2 *ctsR-mcsA* vs Δ*ctsR*		Protein function (specific gene name)
	Adjusted *P* value[c]	Change (*n*-fold)[d]	Adjusted *P* value[c]	Change (*n*-fold)	
lmo0014	0.754	−1.21	**0.038**	−1.62	AA3-600 quinol oxidase subunit I (*qoxB*)
lmo0098	**0.046**	−1.80	0.602	−1.19	Similar to PTS system mannose-specific, factor IID
lmo0175	0.899	1.04	**0.050**	−1.76	Putative peptidoglycan bound protein (LPXTG motif)
lmo0230	**<0.001**	−6.16	0.459	−1.20	Similar to *B. subtilis* McsA (*mcsA*)
lmo0231	**0.001**	−3.46	**0.015**	−4.04	Similar to *B. subtilis* McsB (*mcsB*)
lmo0232	**<0.001**	−4.70	**0.005**	−2.77	Endopeptidase Clp ATP-binding chain C (*clpC*)
lmo0233	**0.014**	−1.70	0.077	−1.49	Similar to DNA repair protein Sms
lmo0362	0.224	−1.71	**0.038**	−1.98	Twin arginine-targeting protein translocase, TatA/E family (*tatA*)
lmo0361	0.292	−1.74	**0.008**	−2.30	Twin arginine-targeting protein translocase (*tatC*)
lmo0373	0.728	−1.16	**0.042**	−1.56	PTS system, lactose/cellobiose family IIC component
lmo0496	**0.013**	−4.04	0.247	−1.79	Similar to *B. subtilis* YnzC protein
lmo0519	0.646	−1.33	**0.034**	−1.69	Similar to multidrug resistance protein
lmo0531	0.172	−2.03	**0.005**	−2.10	Unknown
lmo0600	0.837	−1.19	**0.005**	−2.41	Unknown
lmo0609	0.219	−1.32	**0.008**	−2.08	Similar to *E. coli* phage shock protein E
lmo0770	**0.030**	−1.66	0.455	−1.25	Similar to transcriptional regulator, LacI family
lmo0811	**0.021**	−2.30	0.089	−1.64	Similar to carbonic anhydrase
lmo0822	**0.025**	−1.58	0.133	−1.47	Similar to transcriptional regulators
lmo0944	0.055	−1.42	**0.044**	−1.61	Similar to *B. subtilis* YneR protein
lmo0977	0.517	−1.19	**0.050**	−1.67	Similar to *B. subtilis* YjcH protein
lmo1007	0.173	−2.21	**0.042**	−2.70	Unknown
lmo1096	**0.026**	−1.86	**0.050**	−1.93	Similar to GMP synthetase (*guaA*)
lmo1138	**<0.001**	−6.09	**0.001**	−3.76	Similar to ATP-dependent Clp protease proteolytic component
lmo1258	0.302	−1.18	**0.050**	−1.74	Unknown
lmo1384	0.214	−1.57	**0.015**	−1.92	Unknown
lmo1569	**0.025**	−1.62	0.228	−1.29	Unknown
lmo1583	**0.035**	−1.48	0.938	−1.02	Similar to thiol peroxidases (*tpx*)
lmo1960	0.340	−1.63	**0.036**	−1.77	Similar to ferrichrome ABC transporter, ATP-binding protein (*fhuC*)
lmo1992	0.065	−1.42	**0.050**	−1.52	Similar to alpha-acetolactate decarboxylase
lmo2006	0.155	−1.55	**0.015**	−1.82	Similar to alpha-acetolactate synthase protein, AlsS (*alsS*)
lmo2054	**0.040**	−1.51	0.633	−1.22	Unknown
lmo2114	**0.025**	−1.75	**0.039**	−2.53	Similar to ABC transporter (ATP-binding protein)
lmo2206	**0.030**	−6.72	0.167	−2.00	ATP-dependent Clp protease ATP-binding subunit ClpB (*clpB*)
lmo2205	**0.048**	−3.31	0.902	−1.05	Similar to phosphoglyceromutase 1
lmo2208	**0.007**	−2.18	0.535	−1.14	Unknown
lmo2261	**0.021**	−1.78	0.260	−1.24	Unknown
lmo2369	**0.047**	−1.48	0.932	1.02	Similar to *B. subtilis* general stress protein 13 containing a ribosomal S1 protein domain
lmo2397	**0.025**	−1.57	0.633	−1.10	Similar to NifU protein
lmo2433	0.210	−1.53	**0.001**	−2.76	Similar to acetylesterase
lmo2452	0.285	−1.25	**0.034**	−1.60	Similar to carboxylesterase
lmo2468	**<0.001**	−4.76	0.070	−5.70	ATP-dependent Clp protease proteolytic subunit (*clpP*)
lmo2827	0.201	−1.39	**0.050**	−1.53	Similar to transcriptional regulator, MarR family

[a] All genes that showed evidence for repression by CtsR in either of the two microarray comparisons (i.e., the *L. monocytogenes* parent strain versus Δ*ctsR* or Δ*ctsR* tRNA^{Arg}::pLIV2 *ctsR-mcsA* versus Δ*ctsR* [representing the parent strain expressing *ctsR-mcsA* under an IPTG-inducible promoter]) are listed. Data analyses are based on three independent microarray experiments for the *L. monocytogenes* parent strain versus Δ*ctsR* comparison and on two independent microarray experiments for the Δ*ctsR* tRNA^{Arg}::pLIV2 *ctsR-mcsA* versus Δ*ctsR* comparison; each microarray experiment was performed using an independent RNA isolation from bacteria grown on different days.

[b] Gene names correspond to the gene designations for *L. monocytogenes* strain EGD-e (as listed on the ListiList server [http://genolist.pasteur.fr/ListiList/]); putative operons are in boldface.

[c] Adjusted *P* values of ≤0.05 are in boldface.

[d] Negative changes indicate genes that have lower transcription levels in the *L. monocytogenes* parent strain or in the Δ*ctsR* tRNA^{Arg}::pLIV2 *ctsR-mcsA* strain; positive changes indicate genes have higher transcription levels in the *L. monocytogenes* parent strain or in the Δ*ctsR* tRNA^{Arg}::pLIV2 *ctsR-mcsA* strain.

regulated by CtsR, including seven genes that appear to be directly regulated by both regulators, as supported by identification of both σ^B-dependent promoter sequences and CtsR binding sites upstream of the respective genes. Specifically, CtsR and σ^B coregulate three genes and operons encoding heat shock proteins, including *clpP* and the *clpC* and *clpB* operons (Fig. 7 and 8). Genes and operons indirectly regulated by σ^B and CtsR include a number of genes encoding proteins with confirmed or plausible roles in virulence and stress response. For example, the *gadCB* operon, which encodes a glutamate transporter and decarboxylase important for acid resistance (9), was found to be positively regulated by CtsR and negatively regulated by σ^B (Fig. 7 and 8), and the *tatAC* operon was found to be negatively regulated by CtsR and negatively regulated by σ^B (Fig. 7). Additionally, a number of genes positively regulated by CtsR and negatively regulated by

TABLE 4. Genes identified with lower transcript levels in the Δ*ctsR* strain by microarray analysis[a]

Gene	Differential expression in:				Protein function (specific gene name)
	L. monocytogenes parent strain vs Δ*ctsR*		Δ*ctsR* tRNA^{Arg}::pLIV2 *ctsR-mcsA* vs Δ *ctsR*		
	Adjusted *P* value[c]	Change (*n*-fold)[d]	Adjusted *P* value[c]	Change (*n*-fold)[d]	
lmo0042	**0.048**	1.48	0.870	1.04	Similar to *E. coli* DedA protein
lmo0134	0.193	2.00	**0.015**	2.11	Similar to *E. coli* YjdJ protein
lmo0321	0.168	2.24	**0.015**	2.90	Unknown
lmo0398	**0.046**	2.34	0.765	1.06	Similar to phosphotransferase system enzyme IIA
lmo0683	**0.027**	1.67	0.148	1.47	Similar to chemotactic methyltransferase CheR (*cheR*)
lmo0684	**0.029**	1.53	0.335	1.21	Unknown
lmo0685	**0.032**	1.66	0.199	1.37	Similar to flagellar motor rotation protein MotA (*motA*)
lmo0688	**0.039**	1.73	0.146	1.33	Unknown
lmo0699	**0.028**	1.53	0.811	−1.05	Similar to flagellar switch protein FliM (*fliM*)
lmo0716	**0.040**	1.51	0.260	1.25	Similar to H^+-transporting ATP synthase alpha chain FliI (*fliI*)
lmo0961	0.085	1.68	**0.015**	2.22	Similar to methylated-DNA-protein-cystein methyltransferase
lmo1580	0.303	1.34	**0.039**	1.70	Unknown
lmo1634	0.082	3.50	**0.005**	7.98	Similar to alcohol-acetaldehyde dehydrogenase
lmo2105	0.380	1.74	**0.001**	3.20	Similar to ferrous iron transport protein B
lmo2158	0.085	1.98	**0.003**	3.30	Similar to *B. subtilis* YwmG protein
lmo2230	0.181	2.09	**0.015**	2.55	Similar to arsenate reductase
lmo2340	0.303	1.15	**0.050**	1.90	Similar to *Erwinia chrysanthemi* IndA protein
lmo2362	**0.021**	2.18	0.163	2.91	GadC, amino acid antiporter, involved in acid resistance (*gadC*)
lmo2363	0.072	2.14	**0.001**	3.04	GadB, glutamate decarboxylase (*gadB*)
lmo2637	0.273	1.96	**0.015**	3.07	Unknown, conserved lipoprotein
lmo2638	**0.030**	1.73	0.146	1.92	Similar to NADH dehydrogenase
lmo2832	**0.006**	1.78	0.876	−1.03	Unknown

[a] All genes that showed evidence for repression by CtsR in either of the two microarray comparisons (i.e., the *L. monocytogenes* parent strain versus Δ*ctsR* or Δ*ctsR* tRNA^{Arg}::pLIV2 *ctsR-mcsA* versus Δ*ctsR* [representing the parent strain expressing *ctsR-mcsA* under an IPTG-inducible promoter]) are listed. Data analyses are based on three independent microarray experiments for the *L. monocytogenes* parent strain versus Δ*ctsR* comparison and on two independent microarray experiments for the Δ*ctsR* tRNA^{Arg}::pLIV2 *ctsR-mcsA* versus Δ*ctsR* comparison. As CtsR is a negative regulator, genes down-regulated in the Δ*ctsR* strain are most likely indirectly affected by CtsR.

[b] Gene names correspond to the gene designations for *L. monocytogenes* strain EGD-e (as listed on the ListiList server [http://genolist.pasteur.fr/ListiList/]).

[c] Adjusted *P* values of ≤0.05 are in boldface.

[d] Negative changes indicate genes that have lower transcription levels in the *L. monocytogenes* parent strain or in the Δ*ctsR* tRNA^{Arg}::pLIV2 *ctsR-mcsA* strain; positive changes indicate genes have higher transcription levels in the *L. monocytogenes* parent strain or in the Δ*ctsR* tRNA^{Arg}::pLIV2 *ctsR-mcsA* strain.

σ^B were located in the lmo0675-to-lmo0689 flagellar operon (e.g., *cheR* and *motA*) (Fig. 7); selected genes (i.e., lmo0699 and lmo716) in a downstream flagellar operon (lmo0691 to lmo0718) were also found to be positively regulated by CtsR and negatively regulated by σ^B.

DISCUSSION

L. monocytogenes CtsR was first discovered through the characterization of a naturally occurring *L. monocytogenes* mutant with increased resistance to high pressure; this strain was determined to be missing a single Gly residue in CtsR (28). The *ctsR* null mutant constructed here as a member of a set of otherwise isogenic strains showed increased heat resistance, reduced swarming ability, and reduced efficiency of invasion of Caco-2 cells, further establishing the important role of CtsR in *L. monocytogenes* stress response and virulence (6, 27–29). Increasing evidence suggests that regulatory networks (as opposed to sole reliance on a single regulatory protein) are critical for appropriate expression of stress response and virulence genes in bacteria (34, 46, 49). While *L. monocytogenes* σ^B and PrfA clearly interact to form a network that regulates the transcription of virulence genes in this food-borne pathogen (32, 43, 46), our understanding of the contributions of other transcriptional regulators (e.g., CtsR) to regulatory networks important for virulence and stress response gene expression in *L. monocytogenes* is limited. Initial evidence from our group,

TABLE 5. CtsR-binding sites identified upstream of CtsR-repressed genes

Gene	CtsR-binding sites[a]	Binding site identification
lmo0229	GTCAAAAATAGTCAAAGTCAAN$_{34}$ATG	HMM and DNase I footprinting (49)
lmo0362	*GTCATA*GTTG*GTTAAA*N$_{269}$ATG	HMM
lmo0496	*GTCAAA*TTT*TGACAA*N$_{63}$ATG	HMM
lmo1138	*GGACGA*AAAT*GGACAA*N$_{59}$ATG	HMM
lmo2206	GGTCAAAAAAGGTCAGN$_{28}$ATG	Primer extension analysis and DNase I footprinting (5)
lmo2468	GGTCAATAAAGGTCAAAN$_{61}$ATG	DNase I footprinting (49)

[a] CtsR-binding sites are indicated; underlining indicates CtsR-binding sites in the 5′-to-3′ direction; italics indicate CtsR-binding sites that are in the reverse orientation.

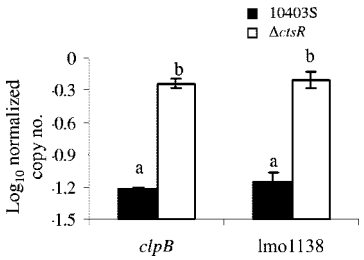

FIG. 6. Transcript levels for *clpB* and lmo1138 in the parent strain (10403S) and the Δ*ctsR* strain grown to log phase in BHI at 37°C. Transcript levels are expressed as log cDNA copy numbers normalized to the geometric mean of cDNA copy numbers for the housekeeping genes *rpoB* and *gap* {i.e., $\log_{10}$ target gene − [($\log_{10}$ *rpoB* + $\log_{10}$ *gap*)/2]}; indicated as "$\log_{10}$ normalized copy no." on the *y* axes). The values shown represent the averages of qRT-PCR assays performed on three independent RNA collections; the error bars show standard deviations. Within a given gene, bars labeled with different letters (a and b) indicate transcript levels that differed significantly ($P < 0.05$; *t* test).

including observations that at least some genes in the CtsR-dependent *ctsR-mcsA-mcsB-clpC* operon show σ^B-dependent transcription under certain environmental-stress conditions (8), suggested that the transcriptional regulators CtsR and σ^B may interact in *L. monocytogenes*. To test the hypothesis that interactions between CtsR and σ^B are important for the expression of virulence and stress response genes in *L. monocytogenes*, we characterized a series of isogenic *L. monocytogenes* mutants, including nonpolar Δ*ctsR*, Δ*sigB*, and Δ*ctsR* Δ*sigB* strains, using phenotypic assays, as well as microarray and qRT-PCR methods. Our results show that (i) a total of 64 genes are regulated by CtsR, either directly or indirectly, including genes important for motility, stress response, and virulence, and (ii) CtsR and its interactions with σ^B contribute to *L. monocytogenes* heat resistance and invasiveness, suggesting an important partnership for these proteins in *L. monocytogenes* stress resistance and virulence.

A total of 64 genes are regulated by CtsR, either directly or indirectly, including genes important for motility, stress response, and virulence. Transcriptomic analyses of the Δ*ctsR* null mutant identified 64 genes as regulated by CtsR, including 10 genes directly repressed by CtsR. While previous studies generally identified small numbers of genes that are directly regulated by CtsR (49), we show that, in addition to genes that are directly repressed by CtsR, the protein also indirectly regulates the transcription of a number of *L. monocytogenes* genes. Identification of these newly recognized CtsR-dependent genes reveals some genetic mechanisms responsible for the phenotypes observed both here and in previous studies of *L. monocytogenes* Δ*ctsR* strains (6, 27–29), including increased resistance to heat, high pressure, and oxidative stresses, as well as reduced motility, virulence, and tissue culture pathogenicity. Correlations between the CtsR-dependent genes identified here and the phenotypic characteristics of Δ*ctsR* null mutants are further detailed below.

Increased heat resistance of *L. monocytogenes* Δ*ctsR* can be explained by the observation that CtsR represses the transcription of a number of genes encoding class III heat shock stress response proteins, including *clpC*, *clpP*, and *clpB* (5, 19, 50), which were all identified as CtsR repressed in our microarray experiments. We also identified lmo1138 and the *tatAC* operon, both of which are directly repressed by CtsR, as novel members of the CtsR regulon. lmo1138 encodes a protein with high homology to other proteins in the large family of ClpP serine proteases, which are critical for the degradation of misfolded proteins. *tatAC* encodes two minimal translocases responsible for the twin arginine translocation (Tat) pathway, which is responsible for the export of fully folded proteins across the cytoplasmic bacterial membrane (13). Identification of these novel CtsR-repressed genes further confirms the importance of CtsR in regulating the transcription of genes that aid *L. monocytogenes* in response to stress conditions that lead to protein denaturation (e.g., heat stress and high pressure) and thus contributes to a better understanding of stress resistance in this important food-borne pathogen.

Interestingly, a number of genes encoding proteins with confirmed or plausible roles in virulence and stress response were also found to be regulated by CtsR. For example, CtsR was found to repress genes (i.e., *qoxB* and *tpx*) that may contribute to *L. monocytogenes* oxidative-stress response, consistent with the observation that *L. monocytogenes* Scott A strains bearing naturally occurring mutations in *ctsR* showed increased resistance to H_2O_2 (27, 29). The *qoxABCD* operon encodes a quinol oxidase, which is important for oxidative-stress response, as supported by observations that a *B. subtilis* strain with a mutation in this operon shows reduced aerobic growth (65). *tpx* encodes a thioperoxidase; interestingly, an *Escherichia coli* O157:H7 *tpx* mutant showed reduced attachment to tissue culture cells, as well as reduced biofilm formation (35). As intracellular bacterial pathogens also experience oxidative stress inside the host cell vacuole (52, 54), it is possible that CtsR-dependent transcription of these genes also contributes to *L. monocytogenes* virulence. The *gadCB* operon appears to be positively regulated by *L. monocytogenes* CtsR, which is of interest, as GadB and GadC have been found to be important for *L. monocytogenes* acid stress survival (9) and thus may contribute to virulence by facilitating passage through the host stomach. While previous studies suggested that CtsR contributes to acid resistance, two *L. monocytogenes* Scott A strains bearing naturally occurring mutations in *ctsR* showed different responses to acid stress, with one mutant (strain 2-1) showing increased acid sensitivity (27) and the other (AK01) showing reduced acid sensitivity (28). Comprehensive evaluation of the acid resistance of isogenic *ctsR* null mutants grown under different conditions and to different growth phases will thus be necessary to pinpoint the specific contributions of CtsR to *L. monocytogenes* acid resistance.

While CtsR's contributions to *L. monocytogenes* virulence have been linked to reduced motility of *L. monocytogenes* Δ*ctsR* mutants, the functional basis of the reduced motility has not previously been identified. Interestingly, our microarray data showed reduced transcript levels for many genes in the lmo0675-to-lmo0689 flagellar operon in the Δ*ctsR* strain (suggesting positive regulation by CtsR), including for *cheR* (encoding a chemotactic methyltransferase) and *motA* (encoding a flagellar rotation motor). Similarly, genes in the downstream flagellar operon (lmo0691 to lmo0718), including *fliM* (encoding a flagellar switch) and *fliI*, also appear to be positively regulated by CtsR. A previous study also reported decreased transcript levels for *flaA*, a struc-

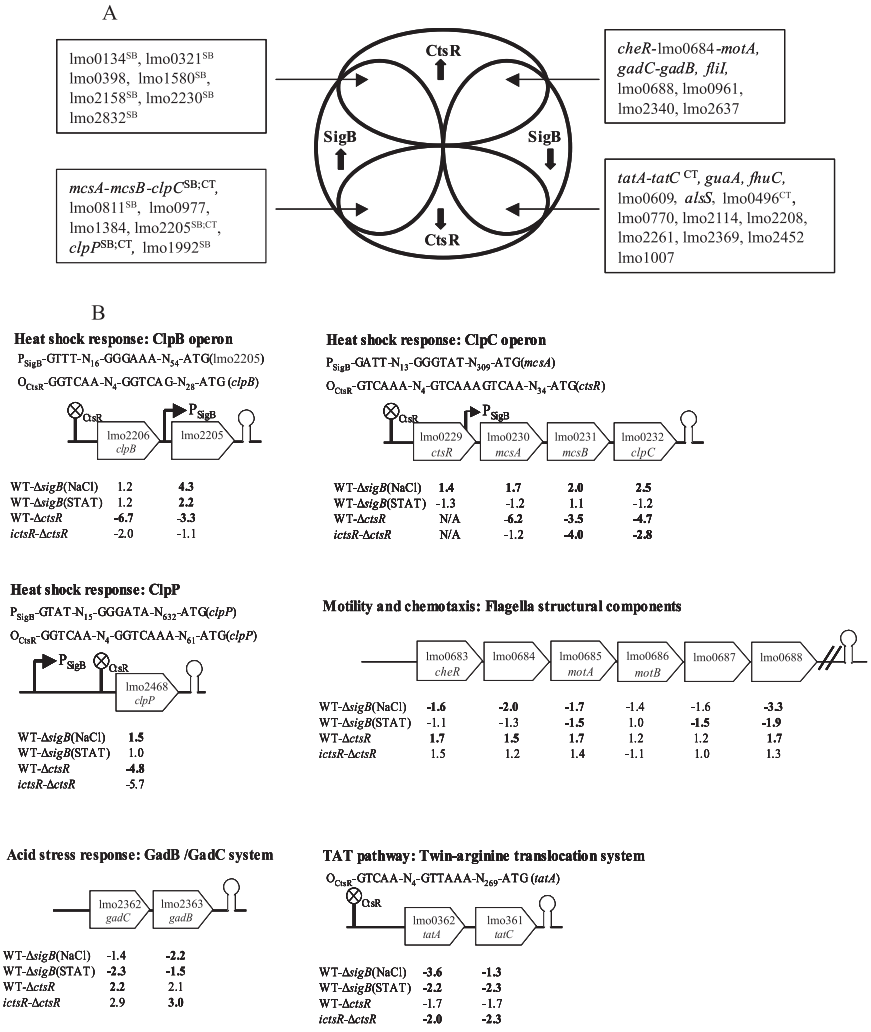

FIG. 7. Genes and operons regulated by both CtsR and σ^B. For both CtsR and σ^B, any genes that showed significant and >1.5-fold differences in transcript levels were considered to be affected by a given regulator. (A) Venn diagram summarizing genes and operons that showed transcript levels that were found in microarray experiments to be significantly affected by either the *ctsR* deletion (this study) or the *sigB* deletion (55a) (microarray data are available under GEO database accession number GSE7492); ↑ and ↓ indicate genes that are up- or down-regulated by a given regulator. Genes preceded by a σ^B consensus promoter or a consensus CtsR binding site are marked with ^{SB} or ^{CT}, respectively. (B) Selected genes and operons regulated by both CtsR and σ^B. For genes that appear to be directly regulated by σ^B and/or CtsR, putative σ^B-dependent promoters and CtsR binding sites are indicated; no putative σ^B-dependent promoters or CtsR binding sites are indicated for the *gadC-gadB* and the lmo0683-to-lmo0688 operons, which appear to be indirectly affected by both of these regulators. Putative terminators (shown as stem-loop structures) are also indicated. Transcript ratios determined in the microarray experiments are indicated below a given gene; differences (*n*-fold) in transcript levels between the parent strain (WT) and the Δ*sigB* strain were determined either in log-phase cells exposed to salt [WT-Δ*sigB*(NaCl)] or in stationary-phase cells [WT-Δ*sigB* (STAT)]; positive numbers indicate that transcript levels were higher in the parent strain (indicating positive regulation by σ^B), while negative numbers indicate that transcript levels were lower in the parent strain (indicating negative regulation by σ^B). For CtsR, differences (*n*-fold) in transcript levels were determined between the parent strain (WT) and the Δ*ctsR* strain grown to log phase (WT-Δ*ctsR*) and between the Δ*ctsR* tRNA^{Arg}::pLIV2 *ctsR-mcsA* and the Δ*ctsR* strain grown to log phase in the presence of IPTG (*ictsR*-Δ*ctsR*); negative numbers indicate that transcript levels were lower in the strain expressing CtsR (indicating negative regulation by CtsR), while positive numbers indicate that transcript levels were higher in the strain expressing CtsR (indicating [indirect] positive regulation by CtsR). Differences (*n*-fold) with adjusted *P* values of <0.05 are in boldface. The lmo0683 to lmo0688 genes shown represent part of a larger operon (lmo0675 to lmo0689).

tural flagellar gene, as well as absence of flagella in a naturally occurring *L. monocytogenes* Scott A *ctsR* mutant (AK01) with a 1-amino-acid deletion in CtsR. Flagella were present on our Δ*ctsR* strain in electron microscopy photographs (data not shown), consistent with its reduced, rather than completely abolished, motility and/or flagellar expression. It is important to note that the parent strain used in our experiments (i.e., *L. monocytogenes* 10403S) is unusual in that it shows motility at 37°C (while most other *L. monocytogenes* strains are motile only at ≤30°C and are nonmotile at 37°C) (62) and that our microarray experiments were conducted using bacteria grown at 37°C, unlike the studies that showed reduced *flaA* transcription in AK01, which used bacteria grown at 30°C (29). It thus appears that CtsR may provide complex contributions to the transcription of multiple flagellar and motility genes and operons, with the possibility of strain- and/or temperature-specific contributions.

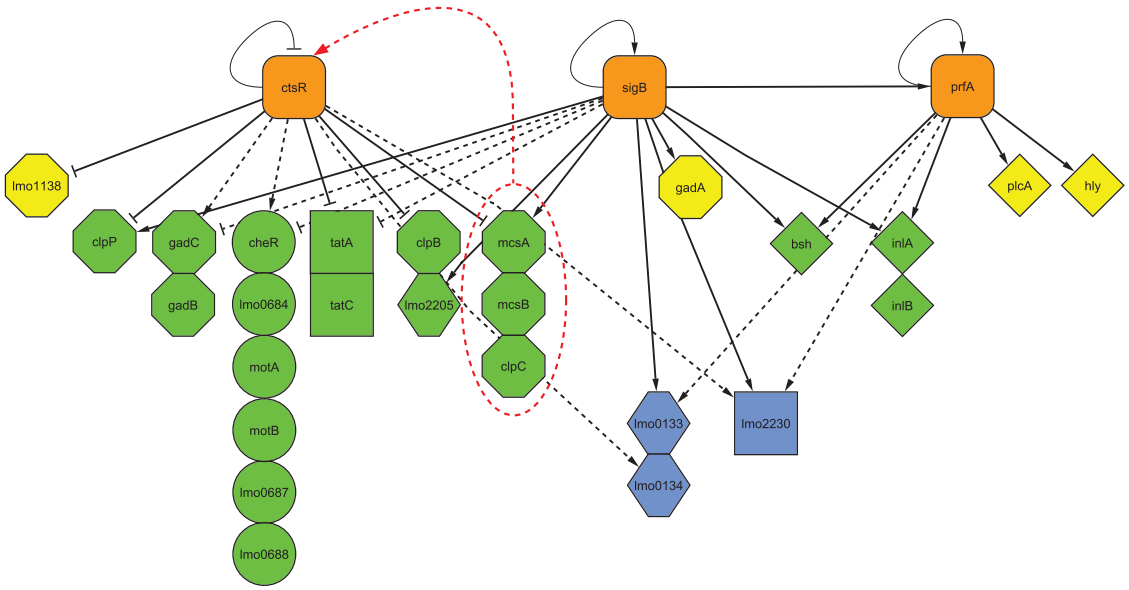

FIG. 8. Partial CtsR, σ^B, and PrfA interaction network. The network is based on CtsR microarray data presented here, σ^B microarray data (55a) (microarray data are available under GEO database accession number GSE7492), and PrfA macroarray data (46). Different symbols were used to represent stress response genes (squares), virulence genes (diamonds), motility genes (circles), stress response genes potentially involved in virulence (octagons), genes of unknown or other function (hexagons), and the genes encoding CtsR, σ^B, and PrfA (orange rounded squares). Solid lines indicate direct regulation of the gene by a given regulator as determined by the presence of a CtsR operator site, σ^B promoter, or PrfA box; dashed lines represent indirect regulation. Target arrows ($\downarrow$) indicate positive regulation by a given regulator (as indicated by higher transcript levels in the parent strain than in the mutant strain); target stops ($\perp$) indicate negative regulation by a given regulator (as indicated by lower transcript levels in the parent strain than in the mutant strain). Loops indicate autoregulation. Color coding was used to identify genes solely regulated by a given regulator (yellow), dually regulated (green), or regulated by all three regulators (blue). Genes arranged in vertical columns represent operons. The red arrow targeting CtsR indicates posttranslational regulation of CtsR by McsA, McsB, and ClpC, based on evidence reported for *B. subtilis* (36, 38).

CtsR and its interactions with σ^B play important roles in *L. monocytogenes* stress resistance and virulence. Both phenotypic and transcriptome data provide clear evidence for the importance of the interplay between σ^B and CtsR in *L. monocytogenes* stress response and virulence. Specifically, statistical analyses of phenotypic data showed that interactions between the *sigB* and *ctsR* null mutations contributed significantly to both heat resistance and invasion efficiency, at least for bacteria grown under some conditions. These interactions were confirmed by transcriptome data, which revealed CtsR- and σ^B-coregulated genes with likely or confirmed roles in heat resistance (*clpC*, *clpP*, and *clpB*) (5, 19). CtsR- and σ^B-coregulated genes with likely roles in invasion and virulence include flagellar and motility genes in the lmo0675-to-lmo0689 operon; contributions of these genes to virulence are supported by previous studies indicating that functional flagella and motility contribute to the ability of *L. monocytogenes* to invade tissue culture cells (14, 55) and to cause infection in a mouse model (55). As the parent strain used in our studies here (10403S) has been reported to show some motility even at 37°C (24, 62), reduced transcription of motility-related genes may also affect invasion efficiency in *L. monocytogenes* 10403S grown at 37°C. Several studies have also suggested that the CtsR- and σ^B-coregulated *clp* genes contribute to *L. monocytogenes* virulence. For example, a Δ*clpC* Δ*clpE* double mutant, as well as Δ*clpB* and Δ*clpC* strains, showed reduced virulence in a mouse model (5, 50, 51) and Δ*clpP* and Δ*clpC* strains showed reduced survival in macrophages (19) and hepatocytes (51), respectively. In addition, *gadC* and *gadB*, which encode proteins important in acid survival and thus may contribute to gastric survival (9), were also found to be coregulated by CtsR and σ^B. In summary, there is considerable evidence that a number of genes with confirmed or likely roles in stress response and virulence are coregulated by CtsR and σ^B.

Our data indicate that CtsR, σ^B, and PrfA form a regulatory network that contributes critically to the regulation of virulence genes, stress response genes with confirmed or plausible roles during infection, and stress response genes without apparent contributions to infection (Fig. 8). While PrfA predominantly regulates classical virulence genes, as defined by the "molecular version of Koch's postulates" (17), including a number of virulence genes coregulated with σ^B, the σ^B regulon includes virulence genes, stress response genes with roles during infection, and stress response genes without apparent roles in infection (30). CtsR, on the other hand, does not appear to regulate any major virulence genes but regulates stress response genes, both with and without apparent roles in infection. The network formed among these three transcriptional regulators suggests the importance of coordinated transcriptional regulation of stress response and virulence genes for transmission of food-borne bacterial pathogens. The interaction among these regulators is not limited to coregulation of genes but also includes interactions that affect the expression of the regulators themselves. CtsR (49), σ^B (1, 56), and PrfA (45) autoregulate their own transcription, and σ^B also contributes to the transcriptional regulation of *prfA* (through the

σ^B-dependent *prfA*P2 promoter (48, 56, 57). σ^B likely contributes to posttranslational regulation of CtsR, as under some conditions, σ^B appears to regulates the transcription of *mcsA-mcsB-clpC* through a σ^B-dependent promoter upstream of *mcsA*, as supported by qRT-PCR data that showed σ^B-dependent transcription of *clpC* in *L. monocytogenes* exposed to energy stress (8). *mcsA-mcsB-clpC* encode proteins that are important for the posttranslational regulation of CtsR; in *B. subtilis*, McsA has been shown to stabilize CtsR, while McsB, when activated by stress, can modify CtsR structure, which not only results in CtsR release from the operator, but also targets the CtsR protein for degradation by the ClpCP protease (36, 38). Interestingly, σ^B-dependent transcription of *clpC* appears to be apparent only under certain environmental conditions; for example, no evidence for σ^B-dependent transcription of *clpC* was found in *L. monocytogenes* exposed to ethanol and salt stress (8). As posttranslational regulation of PrfA, CtsR, and σ^B activities under different environmental-stress conditions and temperatures is also well documented (7, 12, 16, 36, 38, 66), it is clear that we are only beginning to unravel the network formed by these regulators and its contributions to *L. monocytogenes* virulence and stress survival in different intra- and extrahost environments.

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